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(54) Title: REGULATORY NUCLEIC ACID FOR THE ABC1 GENE, MOLECULES MODIFYING ITS ACTIVITY AND THERAPEUTIC USES

(57) Abstract: The present invention concerns a nucleic acid which is capable of regulating the transcription of the ABC1 gene, which is a causal gene for pathologies linked to a dysfunctioning of cholesterol metabolism, inducing diseases such as atherosclerosis. The invention also relates to nucleotide constructs comprising a polynucleotide which encodes a polypeptide or a nucleic acid of interest, placed under the control a regulatory nucleic acid for the ABC1 gene. The invention also relates to recombinant vectors, transformed host cells and nonhuman transgenic mammals comprising a nucleic acid which regulates the transcription of the ABC1 gene or an abovementioned nucleotide construct, as well as methods for screening molecules or substances which are capable of modifying the activity of the regulatory nucleic acid for the ABC1 gene.

These lipoproteins are involved in various metabolic pathways, such as lipid transport, bile acid formation, steroidogenesis or cell proliferation, and also interfere with plasmatic proteinase systems.

HDLs are perfect free cholesterol acceptors, and in combination with cholesterol ester transfer proteins (CETP), lipoprotein lipase (LPL), hepatic lipase (HL) and lecithin-cholesterol acyltransferase (LCAT), play a major role in the reverse transport of cholesterol, i.e. the transport of excess cholesterol in the peripheral cells to the liver, for its removal from the body in the form of bile acid.

It has been demonstrated that the HDLs generally play a central role in the transport of cholesterol from the peripheral tissues to the liver.

Various diseases linked to an HDL deficiency have been described, including Tangier disease, HDL deficiency and LCAT deficiency.

The deficiency involved in Tangier disease is linked to a cellular defect in the translocation of cellular cholesterol, which leads to a degradation of the HDLs.

In Tangier disease, this cellular defect leads to a disruption of lipoprotein metabolism. The HDL particles in Tangier disease, which do not incorporate cholesterol from the peripheral cells, and which are not able to be correctly metabolized, are rapidly eliminated from the body. The plasma HDL concentration in these patients is thus extremely reduced, and the HDLs no longer contribute to the return of cholesterol to the liver. This cholesterol accumulates in these peripheral cells and causes characteristic clinical manifestations such as the formation of orange-colored tonsils. Furthermore, other lipoprotein disruptions such



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Recently, a study was carried out on the segregation of various allelic forms of 343 microsatellite markers distributed over the entire genome and distant from each other by 10.3 cM on average.

The linkage study was carried out on a family which had been well characterized over eleven generations, in which many members are affected by Tangier disease, the family comprising five consanguineous lines.

This study made it possible to identify a region located in the 9q31 locus of human chromosome 9 which is statistically linked to the condition (Rust S. et al., Nature Genetics Vol. 20, September 1998, pages 96-98).

However, the study by Rust et al. only characterizes a wide region of the genome in which impairments are likely to be associated with Tangier disease. The study simply stated that the relevant 9q31-34 region contains ESTs, but no known gene.

It has been shown that a region spanning 1 cM, situated in the 9q31 locus in humans, is generally associated with familial HDL deficiencies (Rust et al., 1999).

Furthermore, it has been shown that a gene encoding a protein of the family of ABC transporters, which is located precisely in the 1 cM region of the 9q31 locus, is involved in pathologies linked to a deficiency in the reverse transport of cholesterol.

For example, it has been shown that the gene encoding the ABC-1 transporter is mutated in patients with affected reverse transport of cholesterol, such as in patients suffering from Tangier disease.



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Other ABC transporters have been associated with neuronal and tumor conditions (US patent No. 5,858,719), or potentially implicated in diseases caused by an impairment of the homeostasis of metals, for example, the ABC-3 protein.

Similarly, another ABC transporter, referred to as PFIC2, seems to be involved in a form of progressive familial intrahepatic cholestasis, this protein being potentially responsible, in humans, for the export of bile salts.

In 1994, a cDNA encoding a novel mouse ABC transporter was identified and referred to as ABC1 (Luciani et al., 1994). This protein is characteristic of the ABC transporters in that it has a symmetrical structure comprising two transmembrane domains linked to a highly hydrophobic segment and to two NBF moieties.

In humans, a partial cDNA comprising the entire open reading frame of the human ABC1 transporter has been identified (Langmann et al., 1999).

It has also been shown that the gene encoding the human ABC1 protein is expressed in various tissues, and more particularly at high levels in the placenta, the liver, the lungs, the adrenal glands and the fetal tissues.

These authors have also shown that the expression of the gene encoding the human ABC1 protein is induced during the differentiation of monocytes into macrophages *in vitro*. Furthermore, the expression of the gene encoding the ABC1 protein is increased when human macrophages are incubated in the presence of acetylated low-density lipoproteins (AcLDLs).

The work of Rust S. et al., 1999, Brooks-Wilson A. et al., 1999, Bodzioch M. et al., 1999, Remaley A. et al., 1999 and of Marcil M. et al., 1999 has shown that patients suffering from Tangier disease and from HDL deficiencies (FHD; familial



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b) The characterization of the regulatory sequences of the ABC1 gene would place at the disposal of persons skilled in the art means capable of allowing the construction, by genetic engineering, and then the expression, of given genes in the cell types in which the ABC1 gene is expressed.

c) Moreover, some portions of the regulatory sequences of the ABC1 gene might constitute high expression-level constitutive promoter sequences, which are liable to enable the construction of novel means for expressing given sequences in cells, completing an already existing set of means.

To date, despite the efforts undertaken, the regulatory sequences of the ABC1 gene have remained totally unknown.

The inventors have henceforth isolated and then sequenced a genomic DNA comprising the first two exons of the ABC1 gene (respectively exon 1A and exon 1B), as well as a non-transcribed region of approximately 2.9 kb, which is located on the 5' side of exon 1A, and which comprises regulation signals for the ABC1 gene.

## BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1:** illustrates a portion of the sequence SEQ ID No. 3, which starts with the nucleotide at position 1 of the sequence SEQ ID No. 3. The position of each of the characteristic binding moieties for various transcription factors is represented by boxes, the designation of the transcription vector specific for the corresponding sequence being indicated above the nucleotide sequence.



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Luciferase values were normalized to β-galactosidase activity and expressed as mean ± SEM.

**Figure 5:** A radiolabelled fragment (-171 to –77 bp) of the human ABC1 promoter was digested with different concentrations of DNaseI in the presence (+) or absence (-) of RAW cell nuclear extract (NE). The G and G+A ladders from Maxam and Gilbert sequence of the radiolabelled fragment are shown. The nucleotide position relative to the transcriptional start site and location of the Sp1, AP1 and EB motifs in the hABC1 promoter are indicated on the right;

**Figure 6A:** illustrates the probes used for the gel shift analysis. Fragment A (100 bp) includes binding motifs for Sp1 and AP1 and the E-box. Fragment EB (27 bp) contains the E-box and fragment EBm (not shown) contains a mutated E-box. In panels B and C, the labelled fragments (fragments A, EB or EBm) used for the gel shift study are shown on top of each gel. Incubation of the radiolabelled probe with RAW cell nuclear extract (NE) is indicated (+);

**Figure 6B:** shows the gel-shift analysis performed by incubating RAW cell nuclear extracts with radiolabelled Fragment A (left), EB (middle) or EBm (right) in the presence or absence of specific competitors (unlabelled fragment A, EB or EBm);

**Figure 6C:** shows supershift analysis of Fragments A or EB with antibodies specific to the amino (N) or carboxyl (C) ends of USF1 and USF2. Arrows indicate



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amount of impurities before the purification. In one embodiment, impurities are present after the purification in an amount of 2 or 3 orders of magnitude less than the amount of impurities before the purification. In another embodiment, impurities are present after the purification in an amount of 4 or 5 orders of magnitude less than the amount of impurities before the purification.

For the purposes of the present description, the expression "nucleotide sequence" can be used to refer indiscriminately to a polynucleotide or a nucleic acid. The expression "nucleotide sequence" encompasses the genetic material itself, and is thus not restricted to the information concerning its sequence.

The terms "nucleic acid", "polynucleotide", "oligonucleotide" and "nucleotide sequence" encompass RNA, DNA or cDNA sequences, or DNA/RNA hybrid sequences of more than one nucleotide, indiscriminately in the single chain form or in the duplex form.

The term "nucleotide" refers to both natural nucleotides (A, T, G, C) and modified nucleotides, which comprise at least one modification such as (1) a purine analog, (2) a pyrimidine analog or (3) a similar sugar, examples of such modified nucleotides being described for example, in PCT application No. WO 95/04 064.

For the purposes of the present invention, a first polynucleotide is considered as being "complementary" to a second polynucleotide when each base of the first nucleotide is paired with the complementary base of the second polynucleotide, the orientation of which is inversed. The complementary bases are A and T (or A and U), or C and G.



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variants with respect to the disease studied is fundamental, since it makes it possible to understand the molecular cause of the pathology.

A "fragment" of a reference nucleic acid according to the invention, will be intended to mean a nucleotide sequence which is shorter in length than the nucleotide sequence of the reference nucleic acid and which comprises a nucleotide sequence which is identical to a portion of the nucleotide sequence of the reference nucleic acid. Such a "fragment" of nucleic acid according to the invention can be, if needed, included in the nucleotide sequence of a second polynucleotide different from the reference nucleic acid. The resulting nucleotide which comprises the "fragment" and the second polynucleotide may have a nucleotide sequence that is longer than, the same length as, or shorter than the nucleotide sequence of the reference nucleic acid. Such fragments comprise, or alternatively consist of, oligonucleotides of lengths ranging from 20 to 25, 30, 40, 50, 70, 80, 100, 200, 500, 1000 or 1500 consecutive nucleotides of a nucleic acid according to the invention.

"Biologically active fragment" of an acid which regulates transcription according to the invention is intended to mean a nucleic acid which is capable of modifying the transcription of a sequence of DNA placed under its control. Such a biologically active fragment comprises a basic promoter and/or a regulatory element, as defined in the present description.

"Regulatory nucleic acid" according to the invention is intended to mean a nucleic acid which activates and/or regulates the expression of a DNA sequence which is selected and placed under its control.



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"Silencer" sequence is intended to mean a DNA sequence included in a regulatory acid, which is capable of decreasing or of inhibiting the transcription initiated by a basic a promoter.

Regulatory elements can be present outside the sequence which is located on the 5' side of the transcription start site, for example, in the introns and the exons, including in the coding sequences.

The terms "basic promoter" and "regulatory element" can be "specific for one or more tissues" if they allow the transcription of a given DNA sequence, placed under their control, such as in certain cells (for example, the cells specific for a tissue), i.e. either exclusively in the cells of certain tissues, or at different levels of transcription according to the tissues.

"Transcription factor" is intended to mean proteins which preferentially interact with regulatory elements of a regulatory nucleic acid according to the invention, and which stimulate or, on the contrary, suppress transcription. Some transcription factors are active in the form of monomers, others being active in the form of homo- or heterodimers.

The term "modification" is directed toward either a positive regulation (increase, stimulation) of transcription, or a negative regulation (decrease, inhibition, blocking) of transcription.

For the purpose of the present invention, the "percentage of identity" between two sequences of nucleotides or amino acids can be determined by comparing two optimally aligned sequences, through a comparison window.



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the databases used can be of peptide or nucleic type, any combination being possible.

For the purposes of the present invention, "high stringency hybridization conditions" are intended to mean the following conditions:

**1 - Membrane competition and PREHYBRIDIZATION:**

- Mixed: 40 µl salmon sperm DNA (10 mg/ml)
  + 40 µl human placental DNA (10 mg/ml)
- Denatured for 5 min at 96°C, then immersed the mixture in ice.
- Removed the 2X SSC buffer and poured 4 ml of formamide mix into the hybridization tube containing the membranes.
- Added the mixture of the two denatured DNAs.
- Incubation at 42°C for 5 to 6 hours, with rotation.

**2 - Labeled probe competition:**

- Added 10 to 50 µl Cot I DNA to the labeled and purified probe, according to the amount of nonspecific hybridization.
- Denatured for 7 to 10 min at 95°C.
- Incubated at 65°C for 2 to 5 hours.

**3 - HYBRIDIZATION:**

- Removed the prehybridization mix.



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The term "transformation also encompasses a situation in which the genotype of a cell has been modified by an exogenous nucleic acid, and in which this cell thus transformed expresses said exogenous nucleic acid, for example, in the form of a recombinant polypeptide or in the form of a sense or antisense nucleic acid.

For the purposes of the invention, "transgenic animal" is intended to mean a nonhuman animal, such as a mammal, in which one or more cells contain a heterologous nucleic acid which has been introduced through human intervention, such as by transgenesis techniques well known to persons skilled in the art. The heterologous nucleic acid is introduced directly or indirectly into the cell or the precursor of the cell, by genetic manipulation such as microinjection or infection with a recombinant virus. The heterologous nucleic acid can be integrated into the chromosome, or can be in the form of DNA which replicates extrachromosomally.

## REGULATORY NUCLEIC ACID FOR THE ABC1 GENE

The inventors have succeeded in isolating a regulatory nucleic acid for the human ABC1 gene from vector libraries of BAC type prepared from human genomic material.

According to the sequence analysis carried out, the inventors have determined that the nucleic acid which regulates the transcription of the ABC1 gene, when it is defined in the broadest way, consists of a polynucleotide comprising, from the 5' end toward the 3' end:

- a nontranscribed region of approximately 2.9 kb located upstream of the transcription start site of the ABC1 gene;



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More precisely, the nucleotide in position 1 of the sequence SEQ ID No. 3 is the nucleotide in position -2893, with respect to the transcription start site of the ABC1 gene.

According to a second aspect, the invention relates to a nucleic acid comprising a polynucleotide which has at least 20 consecutive nucleotides of the nucleotide sequence SEQ ID No. 3, or to a nucleic acid of complementary sequence.

As already specified above, in addition to a 5' non transcribed regulatory region, the nucleic acid which regulates the transcription of the ABC1 gene, of sequence SEQ ID No. 1, also comprises the first exon and the 5' portion of the first intron of the human ABC1 gene.

The first exon of the ABC1 gene, also referred to as exon 1A, is defined as the sequence SEQ ID No. 4.

The sequence of the intron 1a has been partially characterized. The 5' end of intron 1a is defined as the nucleotide sequence SEQ ID No. 6. The 3' end of intron 1a is defined as the sequence SEQ ID No. 7.

The second exon of the human ABC1 gene, also referred to as exon 1B, is defined as the sequence SEQ ID No. 5.

According to a third aspect, the invention relates to a nucleic acid comprising a polynucleotide which has at least 20 consecutive nucleotides of a nucleotide sequence chosen from the sequences SEQ ID No. 3 to 7, or to a nucleic acid of complementary sequence.



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Thus, the various sequences which are characteristic of the binding sites for diverse transcription factors in the sequence SEQ ID No. 3 have been identified by the inventors, in the manner detailed below.

The sequence SEQ ID No. 3 was used as a reference sequence and processed according to the algorithms of the BLAST 2, version 2.10, software, and compared to the data listed in several databases, and the presence, as well as the location, of the various characteristic sites of the sequence SEQ ID No. 3, and, for example, the binding sites for transcription factors, were determined according to methods well known to persons skilled in the art.

Furthermore, a detailed analysis was carried out on the 1.3 kb upstream of the start site, in which a total of 1900 sequences corresponding to binding sites for transcription factors was identified during the first step of the search. After compilation and filtering as described above, only 79 binding sites, specific for 27 different transcription factors, were retained. These sites are presented in Table 1 below.

Table 1 represents the binding sites for the transcription factors identified in the 1318 nt in the 3' portion of the sequence SEQ ID No. 3 according to the invention.

The positions of the start and end nucleotides of each of the binding sites for transcription factors are referred with reference to the numbering of the nucleotides of the sequence SEQ ID No. 3, as represented in Figure 1 (+ strand) or of the nucleotides on the complementary sequence of the sequence SEQ ID No. 3 (- strand)



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TABLE 1 (CONTINUED)

| Transcription factor | Start | End | Strand |
|---|---|---|---|
| AP1(C-JUN) | -731 | -725 | +- |
| PEA3 | -731 | -726 | +- |
| PU.1(NF-JB) | -730 | -725 | - |
| PU.1(NF-JB) | -696 | -691 | - |
| AP2 | -675 | -665 | - |
| AP2 | -671 | -666 | + |
| H-APF-1 | -658 | -652 | + |
| NF-kappaB | -658 | -647 | + |
| GATA1(NF-E1A) | -631 | -626 | +- |
| SP1 | -565 | -559 | - |
| CAC-bf(htbeta) | -557 | -547 | + |
| SP1 | -551 | -542 | - |
| TFIID | -537 | -532 | - |
| NF-E(NF-E1C) | -509 | -505 | - |
| CCAT-bf(CBF) | -508 | -502 | +- |
| GATA1(NF-E1A) | -508 | -504 | + |
| CTF/CBP(NF1) | -507 | -502 | + |
| CP2 | -506 | -502 | - |
| NF-Y | -506 | -502 | + |
| CCAAT-bf(CBF) | -464 | -458 | +- |



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TABLE 1 (CONTINUED)

| Transcription factor | Start | End | Strand |
|---|---|---|---|
| CAC-bf(htbeta) | -240 | -211 | +- |
| SP1 | -238 | -223 | +- |
| GATA1(NF-E1A) | -225 | -219 | + |
| SP1 | -225 | -215 | + |
| SP1 | -221 | -209 | - |
| MYB | -211 | -204 | + |
| GR | -197 | -192 | - |
| SP1 | -189 | -183 | +- |
| MYB | -174 | -167 | - |
| SP1 | -166 | -152 | +- |
| MYC/MAX | -151 | -138 | +- |
| USF 1 and 2 (MYOD) (E-box) | -147 | -142 | + |
| AP1(C-JUN) | -131 | -121 | + |
| SP1 | -100 | -86 | +- |
| CEBP(CEBPA CEBPB) | -90 | -83 | + |
| GR | -80 | -67 | - |
| LXR | -69 | -55 | + |
| TFIID(TBP TATA BOX) | -31 | -26 | + |
| SIF | -3 | 2 | + |



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Figure 1 represents a portion of the sequence SEQ ID No. 3. The first nucleotide in the 5' position of the sequence in Figure 1 is also the first nucleotide in the 5' position of one or the nucleic acid sequences SEQ ID No. 1 and SEQ ID No. 3. In the figure, the binding sites for transcription factors are illustrated with boxes which delimit their respective start and end positions, and their respective designations is indicated above each of the corresponding boxes. The numbering of the nucleotides of the sequence represented in Figure 1 was carried out with respect to the transcription start site, numbered "+1", the nucleotide 5' to the nucleotide +1 being itself numbered "-1".

The description of the characteristics of the binding sites for each of the transcription factors referred to in Figure 1 and Table 1 can easily be found by persons skilled in the art. A short description of some of them is produced below.

**Factor CAC:** The characteristics of a binding site for the factor CAC can be found, for example, in the article by Schuele et al., (1988, Nature, Vol. 332: 87-90), entry No. T00077 of the EMBL database, the article by Mantovani et al., (1988, Nucleic Acids Research Vol. 16: 4299-4313), the article by Catala et al., (1989, Nucleic Acid Research, Vol. 17: 3811-3827) and the article by Wang et al., (1993, Mol. Cell Biol., Vol. 13: 5691-5701). The binding of this factor has been shown on the regulatory regions of several genes, including the promoter for the β-globin gene and the gamma-globin gene. This factor appears to act in cooperation with the glucocorticoid receptor.



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**Factor CP2:**

The characteristics of the binding sites for the factor CP2 can be found, for example, in the articles by Kim et al., (1990), Mol Cell Biol, Vol. 10: 5958-5966 and Lim et al., (1992), J. Biol. Chem. Vol. 268: 18008-18017.

**Factor CTF:**

The binding characteristics of the factor CTF can be found, for example, in the following entries in the Medline database: 88319941, 91219459, 86140112, 87237877, 90174951, 89282387, 90151633, 892618136, 86274639, 87064414, 89263791. The factor CTF/NF-I recognizes the following palindromic sequence: "TGGCANNNTGCCA", which is present in viral and cellular promoters and at the origin of replication of type 2 adenoviruses. These proteins are capable of activating transcription and replication. They bind to DNA in the form of a homodimer.

**Factor E2A:**

The characteristics of a binding site for factor E2A can be found, for example, in the articles corresponding to the following entries in the Medline database: 91160969, 91331308, 91115096, 91117219, 90346284, 89168418, 90150281. This factor binds to a KAPPA-E2 site of the enhancer element of the KAPPA immunoglobulin gene. It forms a heterodimer with the protein ASH1. It belongs to the family of transcription factors of helix-loop-helix type.

**Factor GRα:**

The characteristics of a binding site for the GRα factor can be found, for example, in the following entries in the Medline database: 88264449, 93024441, 89091080, 90319784, 92020837, 90381775, 86298392, 91131612, 86092211,



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**Factor HNF3B:**

Persons skilled in the art may advantageously refer to the article by Overdier et al., (1994, Mol. Cell Biol. Vol. 14: 2755-2766).

**Factor Nfkappa-B:**

Persons skilled in the art may advantageously refer to the articles corresponding to the following entries in the Medline database: 95369245, 91204058, 94280766, 89345587, 93024383, 88248039, 94173892, 91088538, 91239561, 91218850, 92390404, 90156535, 93377072, 92097536, 93309429, 93267517, 92037544, 914266911, 91105848 and 95073993. The factor Nfkappa-B is a heterodimer consisting of a first 50-kDa subunit and a second 65-kDa subunit. Two heterodimers can form a labile tetramer. Its DNA-binding depends on the presence of zinc ($Zn^{++}$). It can be induced by many agents, such as TNF, PKA or PKC. It is generally a regulator of genes involved in responses to infection, inflammation and stress.

**Factor NFY:**

The factor NFY is described, for example, in entry No. P25.208 of the Swissprot database. It is a factor which recognizes a "CCAAT" moiety in promoter sequences such as those of the gene encoding type 1 collagen, of albumin and of β-actin. It is a transcription stimulator.

**Factor PEA3:**

Persons skilled in the art may advantageously refer to the articles corresponding to the following entries in the Medline database: 90059931,



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the c-fos gene.

**Factor RAR:**

Persons skilled in the art may advantageously refer to the articles corresponding to the following entries in the Medline database: 91216109, 92017791, 92127595, 91219411, 92103690, 93321869, 91092269, 91029504, 90242395, 91029504. This factor is a retinic acid receptor. This factor controls cellular functions by directly regulating gene expression. It belongs to the family of hormone nuclear receptors.

**Factor PU:**

Persons skilled in the art may advantageously refer to the articles corresponding to the following entries in the Medline database: 92107189, 93165739, 95317607, 92318913, 92275360, 93028372, 93206099, 90199884, 87257848 and 93275657. This factor binds to the PU box, which is a purine-rich DNA sequence, such as the sequence "GAGGAA", which can act as a lymphoid cell-specific enhancer element.

It is a transcription activation factor which can be specifically involved in the activation or differentiation of macrophages or B cells.

**SITE AP1:**

The characteristics of a binding site for the transcription factor AP1 can be found in various articles corresponding to the following entries in the Medline database: Numbers 89125693, 89252809, 90318391, 91175677, 911458338, 89313776, 88217909, 911662, 91121514, 89017284, 88070595, 90097934,



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for example, in the article by Rosenthal et al., (1990, Nucleic Acids Res., Vol. 18: 6239). This transcription factor induces differentiation of fibroblasts into myoblasts, activates muscle specific promoters and interacts with, and is inhibited by, the protein twist.

**Factor MYC/MAX:**

The characteristics of a binding site for the factor Nyc/MAX can be found, for example, in the following entries in the Medline database: 94040733, 93101610, 92229468, 92112037, 93145325, 93026389, 93157390, 92366516, 93145324 and 91173288. This transcription factor binds to DNA nonspecifically, but also binds to DNA specifically by recognizing the sequence CAC[GA]TG. This factor appears to activate the transcription of genes associated with growth.

**Factor HNF3:**

Persons skilled in the art may advantageously refer to the following entries in the Medline database: 91352065, 91032994, 92345837, 89160814, 91187609, 91160974, 91029477, 94301798 and 94218249. This transcription factor acts as an activator of many genes of the liver, such as AFT, albumin and tyrosine aminotransferase genes, and interacts with regulatory regions which are *cis*-acting with respect to these genes.

**Factor SRY:**

Persons skilled in the art may advantageously refer to the articles corresponding to the following entries in the Medline database: 92132550, 95292338, 95112822, 93049201. This factor is responsible, for example, for initiating male sex determination.



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al., Mol Cell Biol, 1997, 17:18-23). Truncated and splice variants of USF2 which abrogate E-box activity repress the expression of the major histocompatibility complex class I (Howcroft et al., *Mol Cell Biol* (1999) 19:4788-4797), ATPA (Breen et al., J Biol Chem, 1997, 272:10528-10542) and prostaglandin G/H synthase-2 genes. Harris et al (*J Biol Chem*, 2000, 275:28539-28548) have provided evidence that the close proximity between the AP-1 and E-box elements on the FGF-BP promoter facilitates transcriptional repression via interactions between USF1, USF2 and the AP-1 binding proteins. In addition, overexpression of human USF decreases AP-1 dependent transcription in murine teratocarcinoma F9 cells (Pognonec et al., *Oncogene*, 1997, 14:2091-2098) and USF binding to a complex consisting of AP-1 factors, Fra2 and CREB represses the chicken alphaA-crystallin gene (Cvekl et al., 1994, *Mol Cell Biol*, 147363-7376). USF1 and USF2 homodimers inhibit transcription of the ribosomal RNA gene (Sirito et al, *Gen Expr*, 1992, 2:231-240). USF binding motifs have also been shown to act as negative regulatory elements in the promoters of the *Protease Nexin-1* (Erno et al, Mol Cell Neurosci, 1996, 8:28-37) and the HLA-B (Gobin et al., J Immunol, 1999, 163:1428-1434) genes. Interestingly, apolipoprotein CIII, which like ABC1 is involved in lipid metabolism, is also repressed by USF2 (Navantkasattusas et al., Mol Cell biol, 1994, 14: 7331-7339). Finally, USF can both positively and negatively regulate the MLC-2v gene and ribosomal RNA gene transcription. Several studies indicate that USF may also function as a constitutively bound protein that cooperates with basal factors such as $TAF_{II}55$ (Chiang et al., *Science*, 1995, 267:531-536) or inducible factors such as USA (Meisterernst et al., Cell, 1991, 66:981-993), PC5 (Halle et al, J Biol Chem,



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vitellogenin II (Seal et al., Mol Cell Biol, 1991, 11:2704-2717), fatty acid synthase (Casado et al., J Biol Chem, 1999, 274:2009-2013) and the LDL receptor (Sanchez et al., J Biol Chem, 1995, 270:1161-1169) and the LDL receptor related protein (LRP) (Gaeta et al., BBA, 1994, 1219:307-313.)

An preferred characteristic of the regulatory nucleic acid according to the invention, and characteristic of the sequence located upstream of the transcription start site, included in both the sequence SEQ ID No. 1 and in the sequence SEQ ID No. 3, is the presence of eight moieties which are characteristic of a putative binding site for the proteins PPAR. The PPARs, also referred to as peroxisome proliferator-activated receptors, which can be of type $\alpha$,$\delta(\beta)$ and $\gamma$, form a subfamily belonging to the family of nuclear receptor genes. All PPARs are activated by fatty acids and derivatives thereof. For example, the PPAR of type $\alpha$ binds to hypolipidemic fibrates, whereas antidiabetic glitazones are ligands for the PPAR of type gamma. Activation of the PPAR of type $\alpha$ induces pleiotropic effects such as the stimulation of lipid oxidation, the impairment of lipoprotein metabolism and the inhibition of vascular inflammation. Activators of PPAR$\alpha$ increase hepatic absorption and esterification of free fatty acids by stimulating the expression of the fatty acid transport protein and of the acyl-CoA synthetase. In the skeletal muscle and the heart, PPAR$\alpha$ increases mitochondrial absorption of free fatty acids, and their oxidation, by stimulating muscle-specific carnitine palmitoyl transferase I. The effect of fibrates on the metabolism of triglyceride-rich lipoproteins is due to the stimulation of lipoprotein lipase, this stimulation being dependent on PPAR$\alpha$, and to the inhibition of apolipoprotein C-III, whereas the increase in plasmatic cholesterol, in



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increased when human macrophages are incubated in the presence of acetylated low density lipoproteins (AcLDLs).

Without wishing to be bound by any theory, the applicant thinks that the PPAR binding sites identified according to the invention on the regulatory nucleic acid of sequence SEQ ID No. 1 are highly involved in the tissue specific regulation, and in the specific regulation of the cellular metabolic situation, of the ABC1 gene, and that as a result, a regulatory sequence which comprises at least 4, for example, at least 5, 6, 7 or all of the 8 PPAR binding sites (Figure 1)(of the sequence SEQ ID No. 1, and which also comprises a basic promoter element, is useful as a regulatory sequence for a polynucleotide whose expression is desired in the liver, the lungs, the adrenal glands, the monocytes/macrophages, the placenta or the fetal tissues, or for a polynucleotide whose expression is desired in response to a specific stimulation of the cell, in relation with cholesterol metabolism, such as the presence, in the cellular environment, of acetylated low density lipoproteins (Ac LDLs).

In addition, it has been shown according to the invention that a regulatory nucleic acid for the human ABC1 gene, as defined above, which comprises all the abovementioned PPAR sites, is capable of regulating the expression of a coding sequence placed under its control, in a manner which is dependent on the presence of cholesterol in the cellular environment. The results are presented in Example 4 below.

As previously mentioned, the invention concerns a nucleic acid comprising a polynucleotide which has at least 20 consecutive nucleotides of either of the



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nucleotide transcribed being the nucleotide in position 2894 of the nucleotide sequence SEQ ID No. 1.

According to a second embodiment, a biologically active fragment of a transcription-regulating nucleic acid according to the invention comprises both the basic promoter and the proximal regulatory elements, ranging from the nucleotide at position -1 to the nucleotide at position -600, with respect to the transcription start site, the first nucleotide transcribed being the nucleotide in position 2894 of the nucleotide sequence SEQ ID No. 1.

According to a third embodiment, a biologically active fragment of a transcription-regulating nucleic acid according to the invention comprises besides the basic promoter (core promoter) and the proximal 200 bp of the ABC1 gene promoter which is rich in binding sites for transcription factors, *i.e.*, Sp1, AP1, LXR, and E-box, that are likely involved in modulating human ABC1 gene expression.

According to a fourth embodiment, such a biologically active fragment of a transcription-regulating acid according to the invention also comprises, besides the basic promoter (core promoter) and the proximal regulatory elements, other regulatory elements such as the various PPARα sites, and stretches from the nucleotide at position -1 to the nucleotide at position -2894, with respect to the transcription start site, the first nucleotide transcribed being the nucleotide in position 2894 of the nucleotide sequence SEQ ID No. 1.

According to a fifth embodiment, such a biologically active fragment of a transcription-regulating acid according to the invention, which also comprises, besides the basic promoter (core promoter) and the proximal regulatory elements,



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nucleotide sequence SEQ ID No. 1, and is also defined as the sequence SEQ ID No. 6. The 3' end of intron 1A starts at the nucleotide at position 1 and ends at the nucleotide at position 99 of the nucleotide sequence SEQ ID No. 2, and is also identified as the sequence SEQ ID No. 7.

Intron 1B has been partially sequenced. The 5' end of intron 1B starts at the nucleotide at position 259 and ends at the nucleotide at position 357 of the sequence SEQ ID No. 2. This sequence is also identified as the sequence SEQ ID No. 8.

Exon 1B contains the start of the open reading frame of the human ABC1 gene, the nucleotide A of the ATG codon being located in start position at position 94 of the sequence SEQ ID No. 5. Exon 1B encodes the polypeptide of sequence SEQ ID No. 9.

Exons 1A and 1B, as well as introns 1A and 1B, can contain elements for regulating the expression of the ABC1 gene, for example, elements of enhancer type and/or elements of silencer type.

Consequently, a transcription-regulating nucleic acid according to the invention can also contain, besides biologically active fragments of the sequence SEQ ID No. 1, nucleotide fragments, or even all, of the sequences SEQ ID No. 2 to SEQ ID No. 8.

The nucleotide sequences SEQ ID No. 1 to SEQ ID No. 8, as well as fragments thereof, can, for example, be used as nucleotide probes or primers for detecting the presence of at least one copy of the ABC1 gene in a sample, or for amplifying a given target sequence in the regulatory sequence for the ABC1 gene.



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such as at least 4 PPARα sites, or at least 5, 6, 7 PPARα sites or the 8 PPARα sites of the sequence SEQ ID No. 1 or SEQ ID No. 3.

In addition, a tissue specific expression of the polynucleotide of interest can be sought by placing this polynucleotide of interest under the control of a regulatory nucleic acid according to the invention which is capable, for example, of initiating the transcription of this polynucleotide of interest specifically in certain categories of cells, for example, cells from the liver, placenta cells or macrophages.

In general, a regulatory nucleic acid according to the invention can comprise one or more "discrete" regulatory elements, such as enhancer and silencer elements. For example, such a regulatory nucleic acid can comprise one or more potential transcription factor binding sites as defined in Figure 1.

A regulatory acid according to the invention also encompasses a sequence which does not comprise the basic promoter, i.e. the sequence ranging from the nucleotide at position -1 to the nucleotide at position -300, with respect to the transcription start site.

Such a regulatory nucleic acid will then generally comprise a so-called "heterologous" basic promoter, i.e. a polynucleotide comprising a "TATA" box and a "homeobox", which does not originate from the regulatory nucleic acid for the ABC1 gene.

A transcription-regulating nucleic acid comprising all or part of the sequence SEQ ID No. 1 which has been modified, for example, by addition, deletion or substitution of one or more nucleotides, also forms part of the invention. Such



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contain a promoter or a regulatory element with the desired modification, can then be isolated by culturing the transformed host cells having such a construct, in the presence of the given compound, for example, of the given antibiotic.

The reporter gene can also encode any easily detectable protein, for example, an optically detectable protein such as luciferase.

Consequently, an individual of the invention is also a nucleic acid comprising:

a) a transcription-regulating nucleic acid as defined above; and

b) a polynucleotide of interest encoding a polypeptide or a nucleic acid of interest.

According to a first aspect, the polynucleotide of interest whose transcription is desired encodes a protein or a peptide. The protein can be of any nature, for example, a protein of therapeutic interest, including cytokines, structural proteins, receptors or transcription factors. For example, when transcription specifically in certain tissues is desired, such as for example, in liver, macrophage or placenta cells, the transcription-regulating nucleic acid will advantageously comprise a nucleic acid ranging from the nucleotide at position -1 to the nucleotide at position -1318, with respect to the transcription start site of the sequence SEQ ID No. 1 or SEQ ID No. 3.

In this case, the polynucleotide of interest will encode a gene involved in combating inflammation, such as a cytokine receptor, or a superoxide dismutase. If an antitumoral effect is desired, then stimulation of the number and the activation of cytotoxic T lymphocytes specific for a given tumoral antigen will be sought.



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According to a first embodiment, a recombinant vector according to the invention is used to amplify the regulatory nucleic acid according to the invention, which is inserted therein after transformation or transfection of the desired host cell.

According to a second embodiment, they are expression vectors comprising, besides a regulatory nucleic acid in accordance with the invention, sequences whose expression is sought in a host cell or in a given multicellular organism.

According to an advantageous embodiment, a recombinant vector according to the invention will, for example, comprise the following elements:

(1) a regulatory nucleic acid according to the invention;

(2) a polynucleotide of interest comprising a coding sequence included in the nucleic acid to be inserted into such a vector, said coding sequence being placed in frame with the regulatory signals described in (1); and

(3) suitable transcription start and stop sequences.

In addition, the recombinant vectors according to the invention may include one or more origins of replication in the host cells in which their amplification or their expression is desired, markers or selection markers.

By way of examples, the bacterial promoters may be the promoters LacI or LacZ, the T3 or T7 bacteriophage RNA polymerase promoters, or the lambda phage PR or PL promoters.

The promoters for eukaryotic cells may comprise the thymidine kinase promoter of the virus HSV or the mouse metallothionine-L promoter.



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described, for example, by Flotte et al. (1992), Samulski et al. (1989) or McLaughlin BA et al. (1996).

To enable the expression of a polynucleotide of interest under the control of a regulatory nucleic acid according to the invention, the polynucleotide construct comprising the regulatory sequence and the coding sequence may be introduced into a host cell. The introduction of such a polynucleotide construct according to the invention into a host cell can be carried out *in vitro*, according to the techniques well known to persons skilled in the art for transforming or transfecting cells, either in primary culture or in the form of cell lines. The introduction of the polynucleotides according to the invention can also be carried out *in vivo* or *ex vivo*, for preventing or treating diseases linked to a deficiency in the reverse transport of cholesterol.

To introduce the polynucleotides or the vectors into a host cell, persons skilled in the art may advantageously refer to various techniques, such as the calcium phosphate precipitation technique (Graham et al., 1973, Chen et al., 1987), DEAE Dextran (Gopal, 1985), electroporation (Tur-Kaspa, 1896; Potter et al., 1984), direct microinjection (Harland et al., 1985) or DNA-loaded liposomes (Nicolau et al., 1982, Fraley et al., 1979).

Once the polynucleotide has been introduced into the host cell, it can be stably integrated into the genome of the cell. The integration can be carried out at a specific place in the genome, by homologous recombination, or it can be randomly integrated. In some embodiments, the polynucleotide can be stably maintained in the host cell in the form of an episome fragment, the episome comprising



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When the regulatory nucleic acid according to the invention is located, on the polynucleotide construct (or vector), so as to control the transcription of a sequence comprising an open reading frame encoding the ABC1 protein, the vector may be injected into the body of a patient likely to develop a disease linked to a deficiency in the reverse transport of cholesterol, or who has already developed this disease, for example, a patient with a predisposition for Tangier disease, or who has already developed the disease.

Consequently, the invention also concerns a pharmaceutical composition intended for preventing, or for treating individuals affected by, a dysfunction of the reverse transport of cholesterol, comprising a regulatory nucleic acid according to the invention and a polynucleotide of interest encoding the ABC1 protein, in combination with one or more physiologically compatible excipients.

Advantageously, such a composition will comprise the regulatory nucleic acid defined by either of the sequences SEQ ID No. 1 and SEQ ID No. 2, or a biologically active fragment of this regulatory nucleic acid.

A subject of the invention is also a pharmaceutical composition intended for preventing, or for treating individuals affected by, a dysfunction of the reverse transport of cholesterol, comprising a recombinant vector as defined above in association with one or more physiologically compatible excipients.

The invention also relates to the use of a polynucleotide construct in accordance with the invention which comprises a regulatory nucleic acid for the ABC1 gene, as well as a sequence encoding the ABC1 protein, for manufacturing a medicinal product intended for preventing atherosclerosis in various forms or, for



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organ, the modified cell then being reintroduced into the body, or directly in vivo into the appropriate tissue. In this second case, various techniques exist, among which diverse techniques of transfection involving complexes of DNA and of DEAE-dextran (Pagano et al., J. Virol. 1 (1967) 891), of DNA and of nuclear proteins (Kaneda et al., Science 243 (1989) 375), and of DNA and of lipids (Felgner et al., PNAS 84 (1987) 7413), the use of liposomes (Fraley et al., J. Biol. Chem. 255 (1980) 10431), etc. More recently, the use of viruses as vectors for transferring genes has appeared as a promising alternative to these physical techniques of transfection. In this respect, various viruses have been tested for their capacity to infect certain cell populations. For example, retroviruses (RSV, HMS, MMS, etc.), the HSV virus, adeno-associated viruses and adenoviruses.

The present invention thus also relates to a novel therapeutic approach for treating pathologies linked to cholesterol transport, consisting in transferring and in expressing, in vivo, genes encoding ABC1 placed under the control of a regulatory acid according to the invention. The applicant has now advantageously shown that it is possible to construct recombinant viruses which contain a DNA sequence comprising a regulatory nucleic acid according to the invention and a sequence encoding an ABC1 protein involved in cholesterol metabolism, and to administer these recombinant viruses in vivo, and that this administration enables an expression of a biologically active ABC1 protein in vivo, which is stable and effective, and which is without cytopathological effect.

Adenoviruses constitute vectors which are efficient for transferring and expressing the ABC1 gene. For example, the use of recombinant adenoviruses as



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adenovirus or by implantation of a producer cell or a cell which is genetically modified with an adenovirus or with a retrovirus incorporating such a DNA.

The present invention is also advantageous because it makes it possible to induce an expression of ABC1 which is controlled and without harmful effects, in organs which the expression of this protein does not normally concern. For example, a significant release of the ABC1 protein is obtained by implantation of cells which produce vectors of the invention, or which are infected ex vivo with vectors of the invention.

The cholesterol transporter activity produced in the context of the present invention can be of human or animal ABC1 type. The nucleic acid sequence used in the context of the present invention can be a cDNA, a genomic DNA (gDNA) an RNA (in the case of retroviruses) or a hybrid construct consisting for example, of a cDNA into which one or more introns would be inserted. It can also be synthetic or semisynthetic sequences. For example, a cDNA or a gDNA is used. For example, the use of a gDNA allows better expression in human cells. To allow their incorporation in a viral vector according to the invention, these sequences are advantageously modified, for example, by site-directed mutagenesis, for example, for inserting suitable restriction sites. The sequences described in the prior art are not in fact constructed for a use according to the invention, and prior adjustments may prove to be necessary, so as to obtain substantial expressions. In the context of the present invention, use of a nucleic acid sequence encoding a human ABC1 protein is one example. Moreover, it is also possible to use a construct encoding a derivative of these ABC1 proteins. A derivative of these ABC1 proteins comprises,



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herpesviruses (HSV) or retroviruses are used. It is also advantageous to use an adenovirus for a direct administration or for modifying, ex vivo, cells intended for implanting, or a retrovirus for implanting producer cells.

The viruses according to the invention are defective, i.e. they are incapable of replicating autonomously in the target cell. Generally, the genome of the defective viruses used in the context of the present invention is thus lacking at least the sequences required for replication of said virus in the infected cell. These regions can be either removed (totally or partially), or rendered nonfunctional or substituted with other sequences, and, for example, with the nucleic acid sequence encoding the ABC1 protein. For example, however, the defective virus conserves the sequences in its genome which are required for encapsidation of viral particles.

In regards to adenoviruses, various serotypes, whose structure and properties vary somewhat, have been characterized. For example, among these serotypes, in the context of the present invention, type 2 or 5 human adenoviruses (Ad 2 or Ad 5) or adenoviruses of animal origin (see application WO 94/26914) may be used. Among the adenoviruses of animal origin which can be used in the context of the present invention, mention may be made of the adenoviruses of canine, bovine, murine (example: Mav1, Beard et al., Virology 75 (1990) 81), ovine, porcine, avian or simian (example: SAV) origin. For example, the adenovirus of animal origin may be a canine adenovirus, such as a CAV2 adenovirus [Manhattan or A26/61 strain (ATCC VR-800) for example]. For example, in the context of the invention, adenoviruses of human or canine or mixed origin may be used. In addition, the defective adenoviruses of the invention may also comprise ITRs, a sequence



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homologous recombination takes place, for example, after cotransfection of said adenovirus and plasmid into a suitable cell line. The cell line used should, for example, (i) be transformable by said elements, and (ii) have the sequences which are capable of complementing the portion of the genome of the defective adenovirus, such as in an integrated form to avoid the risks of recombination. By way of example of a line, mention may be made of the 293 human embryonic kidney line (Graham et al., J. Gen. Virol. 36 (1977) 59), which comprises, for example, integrated into its genome, the left-hand portion of the genome of an adenovirus Ad5 (12%) or lines which are capable of complementing the E1 and E4 functions, as described, for example, in applications No. WO 94/26914 and WO 95/02697.

Next, the adenoviruses which have multiplied may be recovered and purified according to the conventional techniques of molecular biology, as illustrated in the examples.

As regards the adeno-associated viruses (AAVs), they are relatively small DNA viruses, which integrate into the genome of the cells that they infect, in a stable and site-specific way. They are capable of infecting a broad spectrum of cells, without inducing effects on cell growth, morphology or differentiation. Moreover, they do not seem to be involved in pathologies in humans. The AAV genome has been cloned, sequenced and characterized. It comprises approximately 4700 bases and contains, at each end, an inverted repeat region (ITR) of 145 bases approximately which serves as an origin of replication for the virus. The rest of the genome is divided into two essential regions carrying the encapsidation functions: the left-hand portion of the genome, which contains the rep gene which is involved



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coding regions (gag, pol and env). In retrovirus-derived recombinant vectors, the gag, pol and env genes are generally totally or partially deleted and replaced with a heterologous nucleic acid sequence of interest. These vectors can be prepared from various types of retrovirus, such asfor example, MoMuLV ("murine moloney leukemia virus"; also referred to as MoMLV), MSV ("murine moloney sarcoma virus"), HaSV ("harvey sarcoma virus"), SNV ("spleen necrosis virus"), RSV ("rous sarcoma virus") or the Friend virus.

To construct recombinant retroviruses comprising a sequence encoding the ABC1 protein, placed under the control of a regulatory nucleic acid according to the invention, a plasmid comprising, for example, the LTRs, the encapsidation sequence and said coding sequence is generally constructed, and then used for transfecting a so-called encapsidation cell line which is capable of providing, in trans, the retroviral functions deficient in the plasmid. Generally, encapsidation lines are thus capable of expressing gag, pol and env genes. Such encapsidation lines have been described in the prior art, for example, the line PA317 (US 4,861,719); the line PsiCRIP (WO 90/02806) and the line GP+envAm-12 (WO 89/07150). Moreover, the recombinant retroviruses can comprise modifications in the LTRs so as to eliminate transcriptional activity, as well as extended encapsidation sequences comprising a portion of the gag gene (Bender et al., J. Virol. 61 (1987) 1639). The recombinant retroviruses produced may then be purified by conventional techniques.

To implement the present invention, it is advantageous to use a defective recombinant adenovirus. The results given below indeed demonstrate the



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metabolizing excess cholesterol, and will make it available at the cell surface for its removal by the primary acceptors of membrane cholesterol.

In one embodiment of the present invention, in the vectors of the invention, the sequence encoding the ABC1 protein is placed under the control of a regulatory nucleic acid according to the invention, comprising the regulatory elements which allow its expression in the infected cells and, for example, the regulatory elements of type PPAR.

In another embodiment, the vectors of the invention comprise the sequence encoding the ABC1 protein which is placed under the control of a regulatory nucleic acid comprising a region ranging from the nucleotide at position +108 to the nucleotide at position -2228, with respect to the transcription start site as set forth in sequence SEQ ID No. 1.

Again, in another embodiment, the vectors of the invention comprise the sequence encoding the ABC1 protein which is placed under the control of a regulatory nucleic acid comprising the core promoter sequence and the proximal 200 bp of the ABC1 gene promoter.

As indicated above, the present invention also concerns any use of a virus as described above for preparing a pharmaceutical composition intended for treating and/or for preventing pathologies linked to cholesterol transport.

The present invention also concerns a pharmaceutical composition comprising one or more defective recombinant viruses as described above. These pharmaceutical compositions can be formulated with a view to topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular,



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described above. For example, the invention concerns any population of human cells infected by these viruses. They can be, for example, cells of blood origin (totipotent stem cells or precursors), fibroblasts, myoblasts, hepatocytes, keratinocytes, smooth muscle cells, endothelial cells, glial cells, etc.

The cells according to the invention can be derived from primary cultures. These primary cultures can be sampled by any technique known to persons skilled in the art, and then put into culture under conditions which allow their proliferation. As regards to fibroblasts, they can easily be obtained from biopsies, for example, according to the technique described by Ham [Methods Cell. Biol. 21a (1980) 255]. These cells can be used directly for the infection with the viruses or stored, for example, by freezing, to establish autologous banks, with a view to later use. The cells according to the invention can also be secondary cultures, obtained for example, from pre-established banks (see for example, EP 228458, EP 289034, EP 400047, EP 456640).

The cultured cells are then infected with the recombinant viruses, to confer upon them the capacity of producing a biologically active ABC1 protein. The infection is carried out in vitro according to techniques known to persons skilled in the art. For example, according to the type of cell used and the virus copy number desired per cell, persons skilled in the art can adjust the multiplicity of infection, and optionally, the number of infection cycles carried out. It is clearly understood that the method should be performed under suitable sterile conditions when the cells are intended for an administration in vivo. The doses of recombinant virus used for the infection of the cells can be adjusted by persons skilled in the art according to the



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leads to the adhesion and/or binding of the cells onto the support. Moreover, the cells can either cover the support used, or penetrate inside this support, or both. In the context of the invention, a nontoxic and/or biocompatible solid support may be used. For example, polytetrafluoroethylene (PTFE) fibers or a support of biological origin can be used.

The present invention thus offers an effective means for treating or preventing the pathologies linked to cholesterol transport, such as obesity, hypertriglyceridemia or, in the field of cardiovascular disorders, myocardial infarction, angina, sudden death, heart failure and cerebrovascular accidents.

In addition, this treatment can concern both humans and any animal such as sheep, cattle, domestic animals (dogs, cats, etc.), horses, fish, etc.

## RECOMBINANT HOST CELLS

The invention also concerns a recombinant host cell comprising at least one of the nucleic acids of the invention chosen from sequence SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, and SEQ ID No. 8, for example, a nucleic acid of sequence chosen from SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4 and SEQ ID No. 5.

According to another aspect, the invention also relates to a recombinant host cell comprising a recombinant vector as described above.

Host cells according to the invention are, for example, as follows:



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A first method consists in identifying compounds which modify the expression of the ABC1 gene. According to such a method, cells expressing the ABC1 gene are incubated with a candidate substance or molecule to be tested, and the level of expression of the ABC1 messenger RNA, or the level of production of the ABC1 protein, is then determined.

The levels of ABC1 messenger RNA can be determined with Northern-type gel hybridization, which is well known to persons skilled in the art. The levels of ABC1 messenger RNA can also be determined by methods using PCR or the technique described by WEBB and HURSKAINEN (1996, Journal of Biomolecular Screening, vol. 1 : 119).

The levels of production of the ABC1 protein can be determined by immunoprecipitation or immunochemistry, using an antibody which specifically recognizes the ABC1 protein.

According to another method for screening a candidate molecule or substance which modifies the activity of a regulatory nucleic acid according to the invention, a nucleotide construct as defined above, comprising a regulatory nucleic acid according to the invention and a reporter polynucleotide placed under the control of the regulatory nucleic acid, is used, said regulatory nucleic acid comprising at least one basic promoter and at least one regulatory element from one of the sequences SEQ ID No. 1 and SEQ ID No. 2, a regulatory nucleic acid comprising a region ranging from nucleotides -2228 to + 108 of sequence SEQ ID NO: 1, or a regulatory nucleic acid comprising the core promoter and the 200 pb



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d) comparing the results obtained in c) with the results obtained when the recombinant host cell is cultured in the absence of the candidate molecule or substance to be tested.

The invention also concerns a kit or pack for screening, in vitro, a candidate molecule or substance which is capable of modifying the activity of a regulatory nucleic acid according to the invention, comprising:

a) a host cell transformed with a polynucleotide construct as defined above, comprising a reporter polynucleotide of interest placed under the control of a regulatory nucleic acid according to the invention; and

b) optionally, means for detecting the expression of the reporter polynucleotide of interest.

In one embodiment of the present invention, the reporter polynucleotide of interest is the sequence encoding a luciferase. In this embodiment, the regulatory nucleic acid according to the invention is inserted into a vector, upstream of the sequence encoding the luciferase. It can be for example, the vector pGL3-basic (pGL3-b) sold by the company PROMEGA (Madison, Wisconsin, USA).

In this embodiment, the recombinant vector which comprises the sequence encoding the luciferase, placed under the control of a regulatory nucleic acid according to the invention, is transfected into hepatocellular carcinoma cells, such as the cells of the line HepG2, whose luciferase activity is then determined after culturing in the presence or absence of the candidate substance or molecule to be tested.



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According to such a method, a non-human transgenic animal, for example, a mouse, is treated with a candidate molecule or substance to be tested, for example, a candidate substance or molecule which has been selected beforehand by an in vitro screening method as defined above.

After a given duration, the level of activity of the regulatory nucleic acid according to the invention is determined and compared to the activity of an identical nonhuman transgenic animal, for example, an identical transgenic mouse, which has not received the candidate molecule or substance.

The activity of the regulatory nucleic acid according to the invention, which is functional in the transgenic animal, can be determined by diverse methods, for example, measuring the levels of messenger RNA corresponding to the reporter polynucleotides of interest placed under the control of said regulatory nucleic acid, by Northern-type hybridization or by *in situ* hybridization.

According to one alternative, the activity of the regulatory nucleic acid according to the invention can be determined by measuring the levels of expression of protein encoded by the reporter polynucleotides of interest, for example, by immunohistochemistry, when the polynucleotide reporter of interest comprises an open reading frame encoding a protein which is detectable by such a technique.

For implementing a method for screening, *in vivo*, a candidate substance or molecule which modifies the activity of a regulatory nucleic acid according to the invention, nonhuman mammals may be used, such as mice, rats or guinea pigs or rabbits, whose genome is modified by inserting a polynucleotide construct



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homologous recombination event. Such a technique is described for example, by Mansour et al. (1988, Nature, Vol. 336:348-352).

Next, the positively selected cells are isolated, cloned and injected into 3.5-day old mouse blastocysts, as is described by Bradley (1987, Production and Analysis of Chimaeric mice. In: E.J. Robertson (Ed., Teratocarcinomas and embryonic stem cells: A practical approach. IRL Press, Oxford, page 113). Blastocysts are then introduced into a female host animal, and the development of the embryo is monitored until full term.

According to one alternative, positively selected cells of ES type are brought into contact with 2.5-day old embryos at an 8- to 16-cell stage (*morulae*), as described by Wood et al. (1993, Proc. Natl. Acad. Sci. USA, Vol. 90:4582-4585) or by Nagy et al. (1993, Proc. Natl. Acad. Sci. USA, Vol. 90:8424-8428), the ES cells being internalized so as to extensively colonize the blastocyst, including the cells which give rise to the germ line.

The descendants are then tested to determine those which have integrated the polynucleotide construct (the transgene).

A subject of the invention is thus also a non-human transgenic animal whose somatic and/or germ cells have been transformed with a nucleic acid or a polynucleotide construct according to the invention.

The invention also relates to recombinant host cells obtained from a transgenic animal as described above.

Recombinant cell lines originating from a transgenic animal according to the invention can be established in long term culture starting from any tissue from such



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atherosclerosis, for example, in cholesterol transport, and in the reverse transport of cholesterol.

Firstly, a subject of the invention is also a candidate substance or molecule which modifies the activity of a regulatory nucleic acid according to the invention.

The invention also concerns a candidate substance or molecule characterized in that it increases the activity of a regulatory nucleic acid according to the invention, and for example, of a regulatory nucleic acid comprising the sequence SED ID No.1 or SEQ ID No.3, a region comprising the sequence ranging from nucleotides -2228 to + 108 of sequence SEQ ID NO: 1, or a region comprising the core promoter and the 200 pb proximal of the ABC1 gene promoter

For example, such a substance or molecule which is capable of modifying the activity of a regulatory nucleic acid according to the invention has been selected according to one of the *in vitro* or *in vivo* screening methods defined above.

Thus, an individual whose cholesterol metabolism is affected, for example, an individual affected by Tangier disease, is treated by administering to this individual an effective amount of a compound which modifies the activity of a regulatory nucleic acid according to the invention.

Thus, a patient with a weak activity of the ABC1 promoter can be treated with an abovementioned molecule or substance to increase the activity of the ABC1 promoter.

Alternatively, a patient with an abnormally high activity of the ABC1 promoter can be treated with a compound which is capable of decreasing or blocking the activity of the ABC1 promoter.



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nucleic acid with the gene encoding the transcription factor which binds to the ABC1 promoter decreases the production of this transcription factor, resulting in an increase or a decrease in the activity of the ABC1 promoter, according to whether the transcription factor increases or, on the contrary, reduces the activity of the ABC1 promoter.

The toxicity and the therapeutic efficacy of the therapeutic compounds according to the invention can be determined according to the standard pharmaceutical protocols, in cells in culture or in experimental animals, for example, to determine the lethal dose $LD_{50}$ (i.e. the dose which is lethal for 50% of the population tested) and the effective dose $ED_{50}$ (i.e. the dose which is therapeutically effective in 50% of the population tested).

For all the compounds of therapeutic interest according to the invention, the effective therapeutic dose can be estimated initially from tests carried out in cell cultures *in vitro*.

A subject of the invention is also pharmaceutical compositions comprising a therapeutically effective amount of a substance or molecule of therapeutic interest according to the invention.

Such pharmaceutical compositions can be formulated conventionally, using one or more physiologically acceptable vectors or excipients.

Thus, the compounds of therapeutic interest according to the invention, as well as physiologically acceptable salts and solvates thereof, can be formulated for administration by injection or inhalation, or by oral, buccal, parenteral or rectal administration.



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the addition of one or more nucleotides or of the substitution of one or more nucleotides in said sequence SEQ ID No. 1 or SEQ ID No. 2.

According to one embodiment of a method for detecting an impairment of the transcription of the ABC1 gene in an individual, the genetic alteration is identified according to a method comprising sequencing all or part of the sequence SEQ ID No. 1, or alternatively all or part of at least the sequence SEQ ID No. 2.

Sequencing primers can be constructed in order to hybridize with a given region of the sequence SEQ ID No. 1. Such sequencing primers are for example, constructed so as to amplify fragments of approximately 250 to approximately 300 nucleotides of the sequence SEQ ID No. 1 or of a complementary sequence.

The fragments amplified, for example, by the PCR method, are then sequenced, and the sequence obtained is compared with the reference sequence SEQ ID No. 1 in order to determine whether one or more deletions, additions or substitutions of nucleotides are found in the sequence amplified from the DNA contained in the biological sample originating from the individual tested.

The invention thus also concerns a method for detecting an impairment of the transcription of the ABC1 gene in an individual, comprising:

a) sequencing a nucleic acid fragment which can be amplified with the aid of at least one nucleotide primer which hybridizes with the sequence SEQ ID No. 1 or SEQ ID No. 2 according to the invention;

b) aligning the sequence obtained in a) with the sequence SEQ ID No. 1 or the SEQ ID No. 2;



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b) optionally, the reagents required for carrying out a hybridization reaction.

The nucleic acid fragments derived from any one of the nucleotide sequences SEQ ID No. 1 to 8 are thus useful for detecting the presence of at least one copy of a regulatory nucleotide sequence for the ABC1 gene or of a fragment or of a variant (containing a mutation or a polymorphism) of the latter, in a sample.

The nucleotide probes or primers according to the invention comprise at least 8 consecutive nucleotides of a nucleic acid chosen from the group consisting of the sequences SEQ ID No. 1 to 8, or of a nucleic acid of complementary sequence.

For example, nucleotide probes or primers according to the invention will have a length chosen from 10, 12, 15, 18, 20 to 25, 35, 40, 50, 70, 80, 100, 200, 500, 1000, or 1500 consecutive nucleotides of a nucleic acid according to the invention, such as a nucleic acid of nucleotide sequence chosen from the sequences SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, and SEQ ID No. 8.

Alternatively, a nucleotide probe or primer according to the invention will consist of and/or will comprise the fragments with a length chosen from 12, 15, 18, 20, 25, 35, 40, 50, 100, 200, 500, 1000, and 1500 consecutive nucleotides of a nucleic acid according to the invention, or a nucleic acid chosen from the sequences SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, SEQ ID No. 5, SEQ ID No. 6, SEQ ID No. 7, and SEQ ID No. 8, or of a nucleic acid of complementary sequence.

The definition of a nucleotide probe and primer according to the invention thus encompasses oligonucleotides which hybridize, under the high stringency



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The labeling of the probes is for example, carried out by incorporation of labeled molecules into the polynucleotides by primer extension, or by addition onto the 5' or 3' ends.

Examples of nonradioactive labeling of nucleic acid fragments are described, for example, in French Patent No. FR 78 109 75 or in the articles by Urdea et al. (1988) or Sanchez-pescador et al. (1988).

Advantageously, the probes according to the invention can have structural properties of a type which enables an amplification of the signal, such as the probes described by Urdea et al. (1991), or in European Patent No. EP-0 225 807 (Chiron).

The oligonucleotide probes according to the invention can be used, for example, in Southern-type hybridizations to genomic DNA.

The probes according to the invention can also be used for detecting PCR amplification products or for detecting mismatches.

Nucleotide probes or primers according to the invention can be immobilized on a solid support. Such solid supports are well known to persons skilled in the art, and comprise surfaces of the wells of microtitration plates, polystyrene beds, magnetic beds, nitrocellulose bands or microparticles such as latex particles.

Consequently, the present invention also concerns a method for detecting the presence of a nucleic acid as described above in a sample, said method comprising:

1) bringing one or more nucleotide probes according to the invention into contact with the sample to be tested;

2) detecting the complex possibly formed between the probe(s) and the nucleic acid present in the sample.



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Support matrices onto which oligonucleotide probes have been immobilized at a high density are, for example, described in US Patents No. 5,412,087 and in PCT Application No. WO 95/11995.

The nucleotide primers according to the invention can be used to amplify any one of the nucleic acids according to the invention, and for example, all or part of a nucleic acid of sequences chosen from SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, and SEQ ID No. 5, , or a variant of this nucleic acid.

Another subject of the invention concerns a method for amplifying a nucleic acid according to the invention, and for example, a nucleic acid sequence chosen from SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 4, and SEQ ID No. 5, or a fragment or a variant of this nucleic acid, contained in a sample, said method comprising:

a) bringing the sample, in which the presence of the target nucleic acid is suspected, into contact with a pair of nucleotide primers, of which the hybridization position is located respectively on the 5' side and on the 3' side of the region of the target nucleic acid whose amplification is sought, in the presence of the reagents required for the amplification reaction; and

b) detecting the amplified nucleic acids.

To implement the amplification method as described above, any one of the nucleotide primers described above will advantageously be used.

A subject of the invention is also a pack or kit for amplifying a nucleic acid according to the invention, and for example, all or part of a nucleic acid sequence



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For example, in the case of an analysis by reverse transcription, a pair of primers which have been synthesized from the human ABC1 gene full-length cDNA of sequence SEQ ID No. 10 was used for detecting the corresponding cDNA.

The polymerase chain reaction (PCR) was carried out on matrices of cDNAs corresponding to reverse-transcribed polyA$^+$ mRNAs (Clontech). The reverse transcription into cDNA was carried out with the enzyme Superscript II (GibcoBRL, Life Technologies), according to the conditions described by the manufacturer.

The polymerase chain reaction was carried out according to standard conditions, in 20 µl of reaction mixture, with 25 ng of the cDNA preparation. The reaction mixture was composed of 400 µM of each of the dNTPs, of 2 units of Thermus aquaticus (Taq) DNA polymerase (Ampli Taq Gold; Perkin Elmer), of 0.5 µM of each primer, of 2.5 mM $MgCl_2$ and of PCR buffer. Thirtyfour cycles of PCR (denaturation for 30 s at 94°C, hybridization of 30 s, broken down as follows during 34 cycles: 64°C 2 cycles, 61°C 2 cycles, 58°C 2 cycles and 55°C 28 cycles, and an elongation of one minute per kilobase at 72°C) were carried out after a first denaturation at 94°C for 10 min, in a Perkin Elmer 9700 thermocycler machine. The PCR reactions were visualized on agarose gels by electrophoresis. The cDNA fragments obtained could be used as probes for a Northern blot analysis and could also be used for exactly determining the polynucleotide sequence.

In the case of an analysis by Northern blot, a cDNA probe produced as described above was labeled with $^{32}P$, using the High Prime DNA labeling system (Boehringer), according to the instructions indicated by the manufacturer. After labeling, the probe was purified on a Sephadex G50 microcolumn (Pharmacia),



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**2 - Labeled probe competition:**

- Added 10 to 50 µl Cot I DNA to the labeled and purified probe, according to the amount of repeated sequences.
- Denatured for 7 to 10 min at 95°C.
- Incubated at 65°C for 2 to 5 hours.

**3 - HYBRIDIZATION:**

- Removed the prehybridization mix.
- Mixed 40 µl salmon sperm DNA + 40 µl human placental DNA; denatured 5 min at 96°C, then immersed in ice.
- Added 4 ml of formamide mix, the mixture of the two DNAs and the denatured labeled probe/Cot I DNA to the hybridization tube.
- Incubated for 15 to 20 hours at 42°C, with rotation.

**4 - Washes:**

- One wash at room temperature in 2X SSC to rinse.
- Twice 5 minutes at room temperature, 2X SSC and 0.1% SDS, at 65°C.
- Twice 15 minutes at 65°C, 1X SSC and 0.1% SDS, at 65°C.

After hybridization and washing, the blot was analyzed after overnight exposure in contact with a phosphor screen, which was revealed with the aid of Storm (Molecular Dynamics, Sunnyvale, CA).



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**EXAMPLE 3: Use of IL-1beta-expressing THP-1 macrophages for screening molecules which activate or inhibit the expression of the ABC-1 gene.**

The principle of this assay is that any substance which modifies the synthesis activity of the ABC1 protein has repercussions on the synthesis of IL-1beta.

a) The macrophage cells of the THP-1 lines, which are human monocytic leukemia cells, are a model of differentiated macrophages. These cells were cultured in an RPMI 1640 medium supplemented with 10% of fetal calf serum, in multiwell plates, at a density of 2 105 cells per wells.

b) For the assy per se, the cells were then washed and placed in an RPMI 1640 medium containing 1 mg/ml of purified human albumin fraction IV.

c) The products were added into the extracellular medium. Simultaneously, the cells were then activated by addition of lipopolysaccharides (LPS) for 3 hours, at 1 µg/ml, followed by an incubation of 30 minutes in the presence of ATP at 5 mmol/L.

d) The concentrations of IL-1beta and of control IL-1alpha, tumor necrosis factor alpha (TNFalpha) and IL-6 were determined with ELISA kits, according to the manufacturers' instructions (R&D System; human IL-1beta Chemiluminescent ELISA reference QLB00). The variations in IL-1beta mRNA, which was not supposed to be affected, were evaluated by the Northern blot technique, using the corresponding probe.



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of fetal calf serum (BioWhittaker, Walkersville, MD) was added.

Approximately $1.5 \times 10^5$ cells were distributed into each of the wells of a 12-well (2.5 cm) culture plate, and were cultured until approximately 50 to 70% confluence, and were then cotransformed with 1 μg of the plasmid Sal-Lucif and 0.5 μg of the control vector pBetagel (CloneTech Laboratories Inc., Palo Alto, CA, USA) using the Superfectin Reagent Kit pack (QIAGEN Inc., Valencia, CA, USA). Two hours after adding the DNA, the culture medium was removed, and replaced with complete AMEM (Minimum Essential Medium Eagle's Alpha Modification) medium.

After a duration of twenty hours, the cells were placed in fresh medium such as DMEM (Dulbecco's Minimum Essential Medium), to which 2 μg/ml of glutamine, 100 units/ml of streptomycin and 0.1% of bovine serum albumin (BSA, Fraction V) were added, in the presence or absence of 50 μg/ml of cholesterol (Sigma Chemical Co., St Louis, Missouri, MO, USA).

The cells were recovered 16 hours after the final change of medium using a lysis solution from the Tropix Luciferase Assay Kit pack (Tropix Inc., Bedford, MA, USA).

The cell lysate was divided into aliquot fractions which were stored at -70°C.

Freshly thawed aliquot fractions were used to quantify the proteins, using the MicroBCA Kit pack (Pierce, Rockford, IL, USA), as well as to quantify the luciferase and beta-galactosidase production, using, respectively, the Tropix Luciferase Assay Kit and Galacto-Light Plus Kit packs. The assays were carried out according to the manufacturer's recommendations.



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**EXAMPLE 5: Characterization of the transcription factor binding motifs in the proximal human ABC1 gene promoter.**

**5.1 Materials and Methods**

***5.1.1 Construction of reporter plasmids for Luciferase Assay***

Plasmids containing mutant SP1, AP1, E-box, LXR and deleted E-box fragments were constructed by site-directed mutagenesis using the overlap PCR method and the PXP1 –995 to +120 bp construct (Previato et al., *JBC,* 1991, 266:18958-63) as template. The primers listed below were used to amplify -200 to +44 bp of the human ABC1 promoter. Upper-case letters represent wild-type sequence whereas lower-case letters represent mutant sequence.

| | |
|---|---|
| MDistal SP1F (SEQ ID NO: 14) | 5'TCGCCCGTTTAgGcttgGGcgCCCGGCTC3' |
| MDistal SP1R: | 5'GAGCCGGGcgCCcaagCcTAAACGGGCGA3' |
| MProximal SP1F: (SEQ ID NO: 15) | 5'CAGAGGCCGGGAgGcttgGGcgGGAGGGA3' |
| MProximal SP1R: | 5'TCCCTCCcgCCcaagCcTCCCGGCCTCTG3' |
| MAP1F: (SEQ ID NO: 16) | 5'CGTGCTTTCTGCTGAGgatgcGAACTAC3' |
| MAP1R: | 5'GTAGTTCgcatcCTCAGCAGAAAGCACG3' |
| MEBoxF: (SEQ ID NO: 17) | 5'CGGCTCCtcacggCTTTCTGCTGAGT3' |
| MEBoxR: | 5'ACTCAGCAGAAAGccgtgaGGAGCCG3' |
| DEboxF: (SEQ ID NO: 18) | 5'GCCTCCTTTCTGCTGAGTGACTGA3' |



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cholesterol, 2 µg/ml 22-(R)-hydroxycholesterol (22(R)-Hch), 10µM 9-cis-retinoic acid (9CRA), 10-100 nM estradiol (Sigma, St. Louis, Mo),. 10- 100nM regular insulin (Sigma) or 0.1% ethanol for 24 hours. After harvesting, 10 µl of cell extracts were used for luciferase and β-galactosidase assays by using the Promega dual luciferase assay system (Promega, Madison, WI) or the Tropix Galacto-Light Plus Kits, respectively (Tropix, Bedford MA). The ratio of luciferase activity in relative light units was divided by the β-galactosidase activity to give a normalized luciferase value.

### *5.1.3. Gel Mobility Shift Assay*

Three double-stranded human ABC1 promoter fragments (Fragment A spanning -171 to –71bp; Fragment EB spanning –156 to –130bp and Fragment EB spanning –156 to –130bp) were end-labeled with $_{\alpha}{}^{32}$P-dATP using T4 polynucleotide kinase (Lofstrand, Gaithersburg MD). Nuclear extracts were isolated from unstimulated RAW 264.7 cell and HepG2 cell as well as RAW 264.7 cells after stimulation with the same concentrations of cholesterol and 22(R)-Hch shown above (Paragon Bioservices Inc. Baltimore, MD). One ng (10,000 cpm) of radiolabelled probe was added to 2.5 µg nuclear extract in 20 ul of a 20 mM TRIS gel shift buffer (pH 7.9) containing 60mM KCl, 0.2mM EDTA, 0.5mM DTT, 0.25mM PMS, 1.3mM MgCl, 10% glycerol, 3% Ficoll and 3 µg of double-stranded poly (dIdC) as described (Previato et al., J Biol Chem, 1991, 266:18958-18963) and incubated for 10



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mg/ml) were added. Samples were phenol/chloroform extracted and the aqueous phase was precipitated with 2.5 volumes of 100% ethanol. After a 70% ethanol wash, pellets were dissolved in sequencing gel loading buffer, heated and run on an 8% sequencing gel. Naked DNA was digested with DNaseI as described above except that nuclear extract addition and Protease K treatment were omitted. Maxam-Gilbert sequencing was performed as described in Current Protocols in Molecular Biology (Aubusel et al., *Current Protocols in Molecular Biology*, 1994, 2: 12.1-12.11).

***5.1.5 Western analysis.***

RAW cell nuclear extracts (35 μg protein per lane) from cells stimulated with cholesterol or 22-R-hydroxycholesterol were loaded onto NuPage Bis-Tris 4-12% gradient gels (Invitrogen, Carlsbad, CA) and run according to manufacturer's specifications. Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp, Bedford, MA). Antibodies (2 mg/ml stock) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and were used according to manufacturer's specifications. Anti-USF1 C20X (catalog number sc-229X) antibody was used at a dilution of 1/600 and anti-USF2 antibodies C20X (catalog number sc862X) and N18X (catalog number sc861X) were used at a dilution of 1/1000.

**5.2.1 Analysis of binding motifs in the proximal human ABC1 gene promoter**

To investigate the role of some of the above described transcription factor binding motifs in this region of the human ABC1 gene promoter, *i.e.*, Sp1 (-100 and –166 bp), AP1 (-131 bp), LXR (-69bp) and E-box (-147bp), luciferase reporter



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### 5.2.3 *Mutation of the E-box increases transcription of the human ABC1 gene*

Figure 4 demonstrates that mutation or deletion of the E-box motif increases transcription of the human ABC1 gene in unstimulated RAW cells by approximately 3-fold and also in RAW cells stimulated with cholesterol (c), cis-retinoic acid (CRA) and oxysterol (22OH) by up to 40-fold. Furthermore, mutation or deletion of the E-box in the proximal human ABC1 promoter had no effect on the stimulatory effect of either CRA or oxysterols. Similar findings were demonstrated for unstimulated and stimulated human embryonal kidney 293 cells. These results indicate that the LXR-mediated activation of ABC1 gene transcription by CRA and oxysterols does not require an intact E-box motif and are consistent with binding of a transcriptional repressor to the E-box in the human ABC1 gene.

### 5.2.4 *Nuclear transcription factors bind the E-box in the human ABC1 gene promoter*

DNaseI footprint analysis of the ABC1 proximal promoter revealed protection of the E-box in the presence of RAW nuclear extracts (Fig. 5) indicative of a protein binding to this region. Consistent with the lack of transcriptional effects observed by independently mutating the AP1 and Sp1 motifs (Fig 2B), no protection of these potential binding sites in the human ABC1 promoter was evident by footprint analysis (Fig. 5).

To further demonstrate binding of nuclear transcription factors to the E-box motif we performed gel-shift analysis of the human ABC1 promoter (Fig. 6). The probe utilized in Figure 6B (left) was a 100 bp double-stranded fragment spanning –



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### *5.2.5 USF binds the E-box in the hABC1 gene promoter*

In the human ABC1 promoter, the E-box is flanked by two C's, leading to a sequence of CCACGTGC. This is a perfect match to the consensus motif for the transcription factor USF. To establish that USF is in fact the transcription factor that that binds to the E-box in the hABC1 gene promoter, a gel shift analysis utilizing USF-specific antibodies was performed (Fig. 6C). Using the 100-bp fragment as a probe (Fig. 6C, left panel), it was demonstrated that the E-box gel-shifts some protein in the RAW cell nuclear extract. Addition of anti-USF antibodies against either the amino (N) or carboxy (C) terminus of USF1 or USF2 caused a supershift of the gel-shifted probe, confirming the identity of the E-box binding proteins as USF1 and USF2. Antibodies against other E-box binding proteins including Mad1, Mad2, Mad3, Max, c-Myc, and MyoD as well as Sp1, c-Jun, JunB and JunD did not compete or supershift the DNA-protein gel shift band.

Similar results were obtained by preincubating the 27 bp double-stranded fragment spanning the E-box (EB) with anti-USF antibodies (Fig. 6C; right panel). As with the 100bp gel shift fragment, antibodies specific to other members of the helix-loop-helix family of transcription factors known to also bind the E-box motif did not alter the gel shift band obtained with the 27bp EB probe. No differences have been shown in the gel shift banding patterns obtained when nuclear extracts isolated from unstimulated RAW cells and RAW cells stimulated with cholesterol or oxysterols were incubated with the EB probe .



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**WHAT IS CLAIMED IS:**

1. An isolated nucleic acid comprising a polynucleotide having at least 20 consecutive nucleotides of the nucleotide sequence SEQ ID No. 1, or an isolated nucleic acid of complementary sequence.

2. The isolated nucleic acid according to claim 1, comprising a polynucleotide which has at least 20 consecutive nucleotides of the sequence SEQ ID No. 2, or an isolated nucleic acid of complementary sequence.

3. The isolated nucleic acid according to claim 1, comprising a polynucleotide which has at least 20 consecutive nucleotides of the sequence SEQ ID No. 3, or an isolated nucleic acid of complementary sequence.

4. The isolated nucleic acid according to claim 1, comprising a polynucleotide which has at least 20 consecutive nucleotides of the sequence SEQ ID No. 4, or an isolated nucleic acid of complementary sequence.

5. The isolated nucleic acid according to claim 1, comprising a polynucleotide which has at least 20 consecutive nucleotides of the sequence SEQ ID No. 5, or an isolated nucleic acid of complementary sequence.

6. The isolated nucleic acid according to claim 1, wherein said nucleic acid modifies the transcription of a polynucleotide placed under its control.

7. The isolated nucleic acid according to claim 6, wherein said isolated nucleic acid is a polynucleotide comprising a sequence ranging from the nucleotide at position -1



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respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

13. The isolated nucleic acid according to claim 6, wherein said isolated nucleic acid activates the transcription of a polynucleotide of interest placed under its control.

14. The isolated nucleic acid according to claim 6, wherein said isolated nucleic acid inhibits the transcription of a polynucleotide of interest placed under its control.

15. An isolated nucleic acid having at least 80% nucleotide identity with an isolated nucleic acid according to claim 1.

16. The isolated nucleic acid according to claim 15, wherein said isolated nucleic acid modifies the transcription of a polynucleotide placed under its control.

17. The isolated nucleic acid according to claim 15, wherein said isolated nucleic acid is a polynucleotide comprising a sequence ranging from the nucleotide at position -1 to the nucleotide at position -300, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

18. The isolated nucleic acid according to claim 15, comprising a polynucleotide ranging from the nucleotide at position -1 to the nucleotide at position -600, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

19. The isolated nucleic acid according to claim 15, comprising a polynucleotide ranging from the nucleotide at position -1 to the nucleotide at position -200, with respect to



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25. The isolated nucleic acid according to claim 24, wherein said isolated nucleic acid is a polynucleotide comprising a sequence ranging from the nucleotide at position -1 to the nucleotide at position -300, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

26. The isolated nucleic acid according to claim 24, wherein said isolated nucleic acid is a polynucleotide comprising a sequence ranging from the nucleotide at position -1 to the nucleotide at position -200, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

27. The isolated nucleic acid according to claim 24, comprising a polynucleotide ranging from the nucleotide at position -1 to the nucleotide at position -600, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

28. The isolated nucleic acid according to claim 24, comprising a polynucleotide ranging from the nucleotide at position -1 to the nucleotide at position -2894, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.

29. The isolated nucleic acid according to claim 24, comprising a polynucleotide ranging from the nucleotide at position +120 to the nucleotide at position -995, with respect to the first nucleotide transcribed, which is located at position 2894 of the nucleotide sequence SEQ ID No. 1.



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38. A host cell transformed with a recombinant vector according to claim 35.

39. A non-human transgenic mammal wherein at least one of said mammals cells chosen from somatic cells and germ cells have been transformed with at least one isolated nucleic acid according to one of claims 1 to 34.

40. A non-human transgenic mammal wherein at least one of said mammals cells chosen from somatic cells and germ cells have been transformed with a recombinant vector according to claim 35.

41. A method for screening a substance or a molecule which modifies the transcription of the polynucleotide which is a constituent of the isolated nucleic acid according to claim 33, comprising:

a) culturing a host cell transformed according to one of claims 37 and 38;

b) incubating the transformed host cell in the presence of the candidate substance or molecule;

c) detecting the expression of the polynucleotide of interest;

d) comparing the detection results obtained in c) with the detection results obtained by culturing the transformed host cell in the absence of the candidate molecule or substance.

42. A kit for screening, *in vitro*, a candidate molecule or substance which modifies the transcription of the polypeptide of interest which is a constituent of the isolated nucleic acid according to claim 33, comprising:

a) a host cell transformed according to one of claims 37 and 38;



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46. The substance according to claim 45, wherein said substance comprises at least one molecule which modifies the transcription of a polynucleotide of interest which is a constituent of the isolated nucleic acid according to claim 33.

47. The substance according to claim 45, wherein said substance is selected according to the method of claim 41 or the method of claim 43.

48. A pharmaceutical composition comprising, as active principle, at least one substance or a molecule according to one of claims 45 to 47.

49. The pharmaceutical composition according to claim 48, wherein said composition is used for treating a disorder chosen from hypercholesterolemia and atherosclerosis.

50. A substance according to one of claims 45 to 47, as an active principle of a medicinal product.

51. A method for detecting an impairment of the transcription of the ABC1 gene in an individual, comprising:

a) extracting the total messenger RNA from a biological material originating from the individual to be tested;

b) quantifying the ABC1 messenger RNA present in said biological material:

c) comparing the amount of ABC1 messenger RNA obtained in b) with the amount of ABC1 messenger RNA expected in a normal individual.



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b) detecting the complex formed between the candidate molecule or substance and the candidate molecule or substance.

56. A kit or pack for screening a candidate molecule or substance which modifies the transcription of the polynucleotide of interest which is a constituent of the isolated nucleic acid according to claim 33, comprising:

a) at least one isolated nucleic acid according to one of claims 1 to 34 or a recombinant vector according to claim 35;

b) optionally, the means required for detecting the complex formed between the candidate molecule or substance and said isolated nucleic acid.



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-2781
NKX2.5
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-2721 S8 GATA
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-2661
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-2601
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-2541 IK2 LYF1
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-2481
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-2421 LMO2COM/MYOD/ ΔEF1
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-2361 LYF1 ΔEF1 NKX2.5
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-2301 NFY/CAAT
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-2241 MZF1
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-2181 MZF1 HFH2/SRY/
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-2121 EVI1 CREBP1/VBP NKX2.5
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-2061
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-2001
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**Figure 1**



BNSDOCID: <WO_____0183746A2_I_>

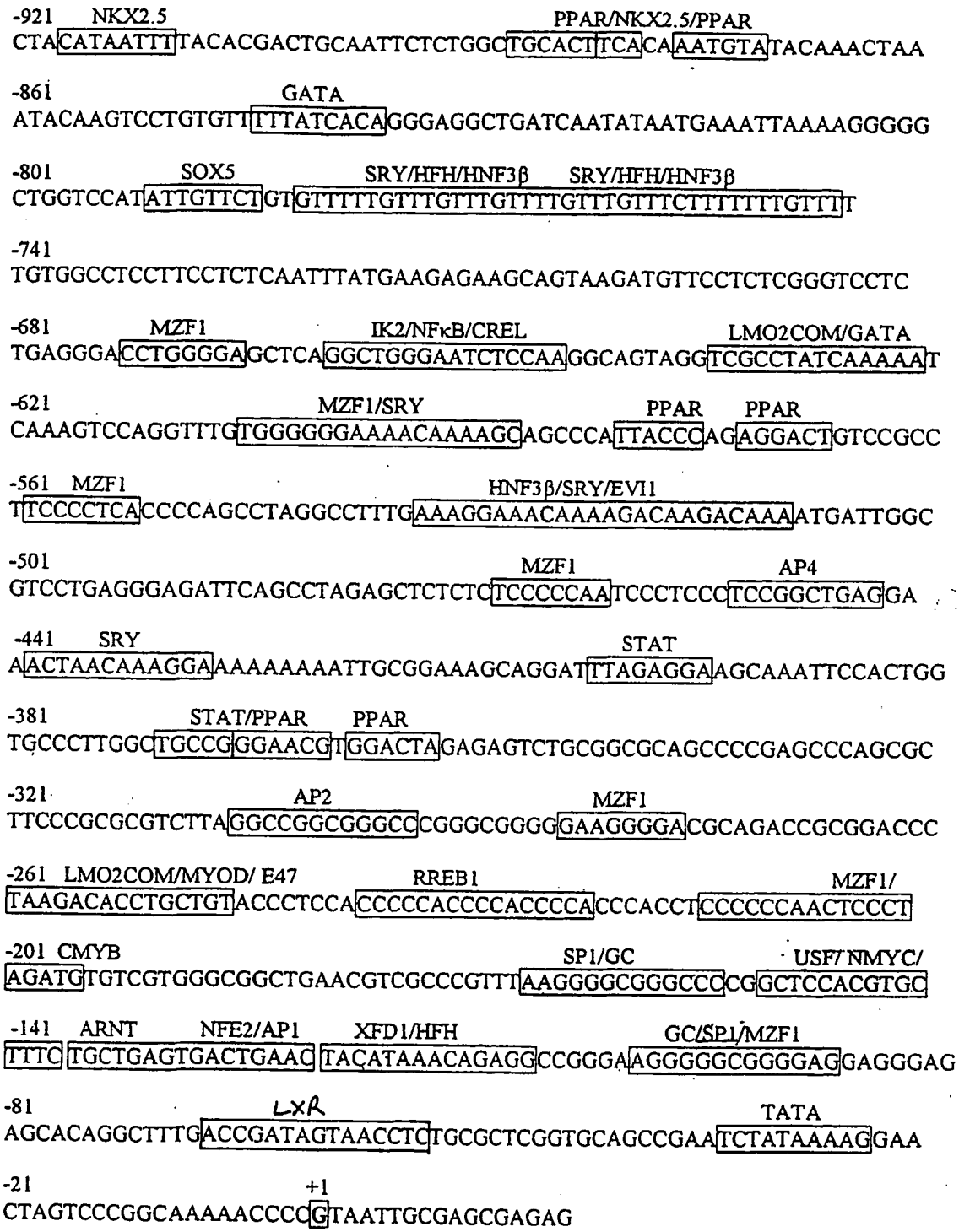


**Figure 1 (suite 2)**



BNSDOCID: <WO_____0183746A2_I_>

FIGURE 3

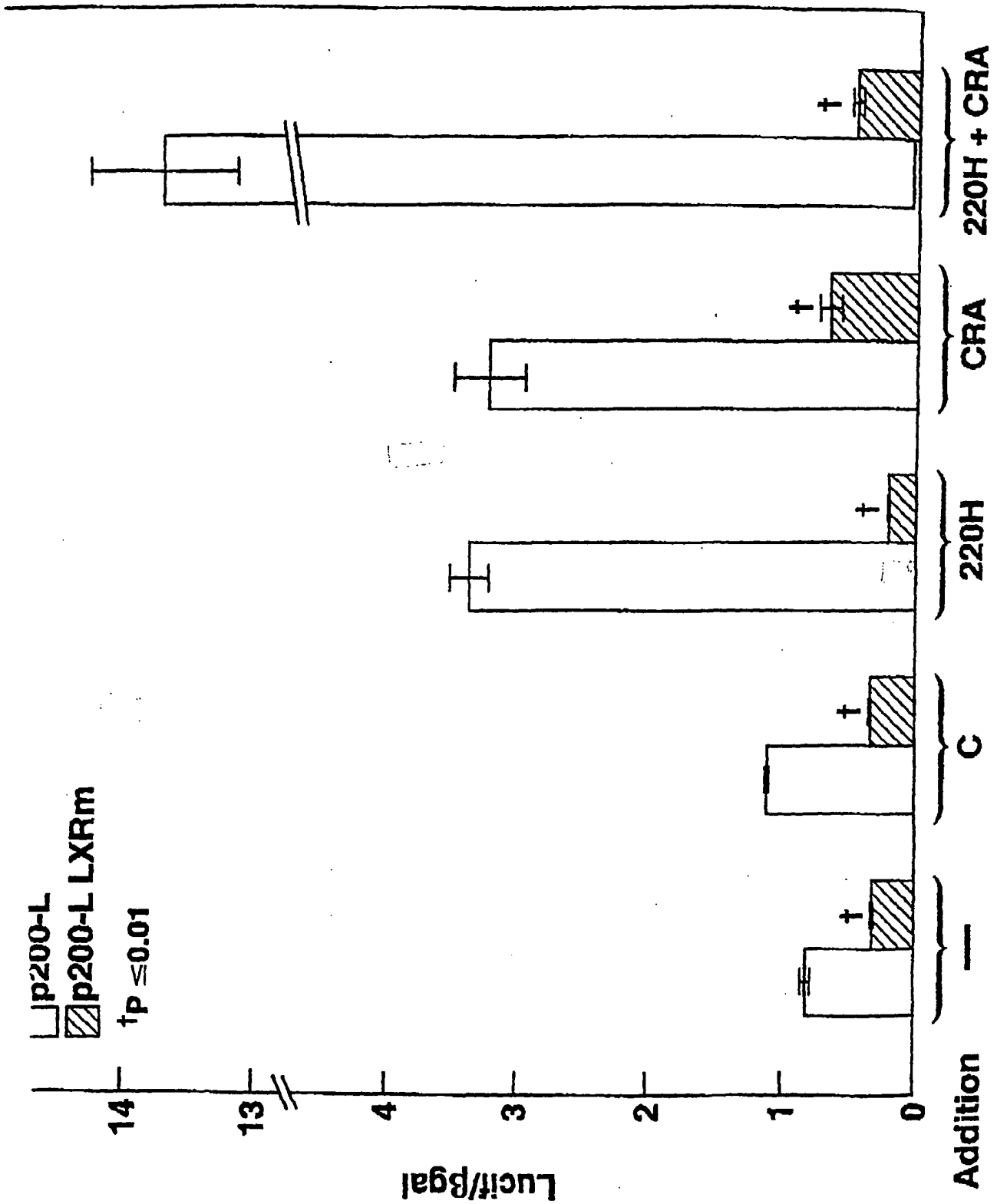




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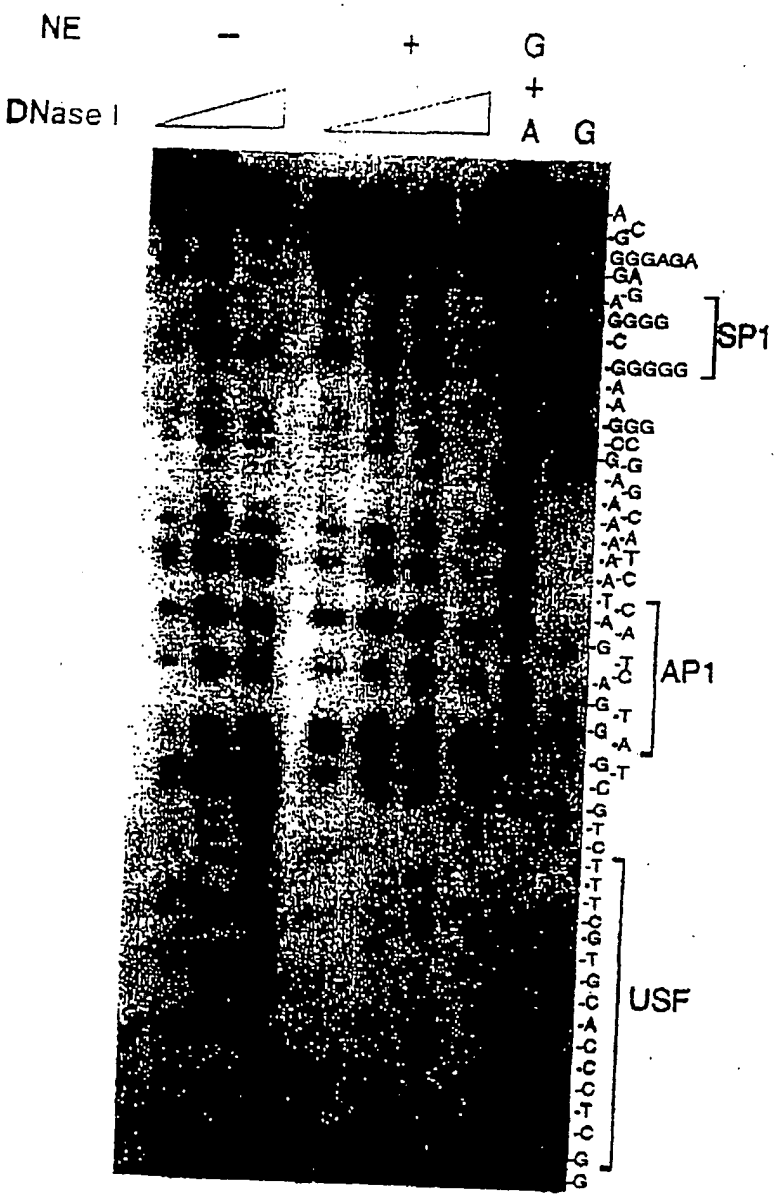
FIGURE 5



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(54) Title: REGULATORY NUCLEIC ACID SEQUENCES OF THE ABC1 GENE

(57) Abstract: The present invention concerns a nucleic acid which is capable of regulating the transcription of the ABC1 gene, which is a causal gene for pathologies linked to a dysfunctioning of cholesterol metabolism, inducing diseases such as atherosclerosis. The invention also relates to nucleotide constructs comprising a polynucleotide which encodes a polypeptide or a nucleic acid of interest, placed under the control a regulatory nucleic acid for the ABC1 gene. The invention also relates to recombinant vectors, transformed host cells and nonhuman transgenic mammals comprising a nucleic acid which regulates the transcription of the ABC1 gene or an abovementioned nucleotide construct, as well as methods for screening molecules or substances which are capable of modifying the activity of the regulatory nucleic acid for the ABC1 gene.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Inte ional Application No
PCT/EP 01/05488

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